

The University of Chicago

THE RESPIRATORY CATALYSTS OF  
THE ROUS SARCOMA

A DISSERTATION SUBMITTED TO THE FACULTY  
OF THE DIVISION OF THE PHYSICAL SCIENCES  
IN CANDIDACY FOR THE DEGREE OF DOCTOR  
OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

1932

By

DAVID OSCAR ROSBASH

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# I

## INTRODUCTION

The metabolism of the Rous sarcoma is, in many respects, different from that of the muscle tissue in which it grows. The impairment of the capacity of tumor tissue to oxidize the rearrangement products of glucose to carbon dioxide is such that lactic acid tends to accumulate even in the presence of an adequate supply of air.<sup>1</sup> The capacity to reduce methylene blue is diminished to an extent not observed in normal tissue.<sup>2</sup>

The proteolytic enzymes of respiring tissues are of two types, the one maximally active at a pH lower than the other.<sup>3</sup> The lowered oxidizing power in tumor tissue may favor the accumulation of inhibitors capable of inactivating the secretory enzymes of the normal inhibitors of the fibrinolytic system. Goldschmidt-Leitz<sup>5</sup> and Voegtli<sup>6</sup> have shown the significance of the suggestion, the cryptic type of enzyme, is, in comparison to the concentration of the enzyme.<sup>7</sup> No significant change in either the activity of the proteolytic enzymes present in normal tissue observed in tumor tissue.<sup>7,8</sup> The sulfhydryl content of tumor tissue may be lower, rather than higher, than that of normal tissue. The pH of the cytoplasm and nucleus of tumor cells is not materially different from that of normal cells.<sup>10</sup> The reported production by malignant tissue of a further, normally absent, type of proteolytic enzyme characterized by fibrin-dissolving properties,<sup>11</sup> not derived from the leucocytes present, has not been confirmed at this writing.

Disturbances in the lipolytic activity of tumor cells have been suggested<sup>12</sup> but have not been established.

Attempts have been made to explain the differences in the character of the metabolic processes maintained in normal and tumor tissue upon a basis of difference in chemical composition. The analyses undertaken have, unfortunately, been concerned almost altogether with the building materials of the cells rather than with their catalytic constituents. Differences have been observed in water and lipid content,<sup>13</sup> and in the amount and proportion of Ca,<sup>14</sup> Mg,<sup>15</sup> Na,<sup>16</sup> K,<sup>17</sup> etc.,<sup>18</sup> present. None of the differences observed appear to clarify the underlying reasons for the differences in metabolism maintained.

Iron has been believed to function in the catalysis of respiration through the activation of molecular oxygen since the first groping theories of Traube and Verworn were enunciated. The concept was first crystallized into a form susceptible of experimental test by Warburg, in 1921.<sup>19</sup> It was recognized at that time that copper was, perhaps, better adapted to function as an oxidase but the significance of the universal distribution of copper in living matter, established as early as 1832 by Sarzeau,<sup>20</sup> was not yet fully appreciated. Imagination was dominated by the hemoglobin picture; the activation of oxygen was interpreted along the lines known to govern its transport and storage in the blood and tissue.

The identity of Warburg's so-called "respiration ferment" with the non-catalytic, intracellular pigment "cytochrome," was suggested by Keilin in 1928,<sup>22</sup> and established by Shibata and Tamiya in 1930.<sup>21</sup> Keilin's own "indophenol oxidase" of heart muscle<sup>22</sup> would appear to contain copper rather than iron. It is obtained from the tissue richest in copper. It is poisoned by



cyanide but not by pyrophosphate, the basis proposed by Warburg for the differentiation of copper from iron in the catalysis of the oxidation of cysteine, in vitro.<sup>23</sup> Iron, in many of its compounds, is an effective oxidase. It is doubtful whether iron behaves as an oxidase in uninjured mammalian tissue.

The possibility that respiration and metabolism might be motivated by a balance between the catalytic potentialities of more than one metal was suggested by Locke and Main in 1930.<sup>24A</sup> As the result of a study of the respiratory substances of the bacteria, the existence was postulated of two general types of catalyst: the one motivated by cationic, oxidizing copper and the other by anionic, reducing iron. The former group embraced the oxidases and the proteases of the trypsin type, the latter the dehydrogenases and proteases of the cathepsin type. It was stated that each of the two types of catalyst "is the perfect foil of the other; combined, the summation is inactive; slightly separated, as when embedded in different portions of the cellular structure, the ions bear the relations of the two poles of an electrolytic cell."<sup>24B</sup> This concept of a metabolism motivated by a reaction potential contributed to by positively charged, oxidizing copper, embedded in the protoplasm and behaving toward similarly embedded, anionic, reducing iron as the oxidizing electrode of an electrolytic cell behaves toward the reducing electrode, already strengthened by studies of the copper:iron balances in the plasma in health and disease,<sup>25</sup> receives further support in the material here presented.

## II

### The Comparative Copper and Non-Hemoglobinous Iron Contents of the Tissues of Normal and Sarcomatous Chickens

The chickens were pure-bred Barred Rock pullets of

varying weight. They were given access to sunlight and to open air and kept under as healthful conditions as possible. The original sarcoma inoculum was obtained from Dr. Peyton Rous as a dried preparation which had been sealed in ampules in 1925.<sup>26</sup> It gave rapid takes in the chickens injected. The tumor tissues produced were examined by Dr. Edwin F. Hirsch and found to have the structure of true spindle cell sarcomas. The tumor was reproduced in other chickens by the injection of 1 cc. of a 10% suspension of the tumor substance in Ringer's solution.

Food was withheld from the chickens for 14 to 16 hours before death by bleeding. The tissues were removed and prepared for analysis immediately after death with precaution against metal and other contaminations. Weighed amounts were then triturated for one and a half hours with one volume of water and two volumes of 20% trichloroacetic acid and the extracts clarified by centrifugation and recentrifugation for 25 and 10 minutes respectively at 1500 r.p.m. The analyses were carried out according to methods developed by Locke, Main and Rosbash for the determination of copper and iron in plasma.<sup>25</sup> 1200 determinations were made in approximately 300 preparations of tissue.

The amounts of copper and non-hemoglobinous iron recovered in the extracts used were within 85-90% of the amounts of copper and non-hemoglobinous iron originally present in the tissues extracted. The yellow color in the extracts of heart, liver, and kidney did not interfere with the copper determinations. The brown color of the extracts of necrotic tumor tissue was removed by digestion with sulfuric acid and perhydrol. Precaution was taken against unnecessary agitation in the preparation of the trichloroacetic acid extracts of tumor tissue. The extracts were, otherwise, cloudy in character and unsuitable for analysis.



The iron extracted from tissue by 10% trichloroacetic acid, in part, is derived from the disintegration of hemoglobin. Determinations of the total iron content of erythrocytes, tissues, and hemoglobin-free bacterial preparations and of the fractional amounts extracted by trichloroacetic acid following saturation with air, carbon monoxide and nitric oxide, respectively, have not only indicated that corrections can be made for iron derived from hemoglobin but that the corrected values so obtained can be used as an index of "active" or catalytic iron content.<sup>27</sup>

Table I compares the copper and non-hemoglobinous iron of the tissues of normal and tumor-bearing chickens kept both in air and in air containing 0.05-.07 per cent of carbon monoxide. The first group of figures presents micrograms of copper extractable by 10 per cent trichloroacetic acid per gram of moist tissue. The second group records iron values, the third copper:iron ratios, and the fourth, the comparative total weights of the different tissues, per kg. of chicken.

The brains of the normal chicken examined contained an average of 2.1 micrograms of extractable copper per gram and an equivalent amount of non-hemoglobinous iron. The copper/iron ratio was, therefore, 1/1. The heart tissue contained considerably more of each metal but approximately the same proportion. The liver, kidney, and muscle were characterized by the much lower ratio of 1/3. They differed in the amount rather than in the proportion of the catalysts present. The liver, with its high metabolic rate, indicated by the fact that it is the warmest large organ in the body, had approximately twelve times as much of both copper and iron as the inactive breast muscle.

The weights of the different organs, per kg. of chicken, were not affected by maintenance in an atmosphere containing

TABLE I

THE COPPER AND NON-HEMOGLOBINOUS IRON CONTENT OF THE TISSUES OF NORMAL AND TUMOR-BEARING CHICKENS AND CHICKENS KEPT IN AIR CONTAINING 0.05 TO .07 PER CENT CARBON MONOXIDE

| Freshly removed                               | Brain | Heart | Liver | Kidney | Muscle | Sarcoma  | RBC | Plasma |
|-----------------------------------------------|-------|-------|-------|--------|--------|----------|-----|--------|
| micrograms of copper per gram                 |       |       |       |        |        |          |     |        |
| Normal Hens                                   | 2.1   | 3.3   | 3.6   | 4.1    | 0.3    | ---      | --- | .09    |
| Tumor-bearing Hens                            | 2.1   | 3.1   | 3.2   | 2.0    | 0.3    | 0.3-0.7* | .6  | .17    |
| Hens kept in CO                               | 2.1   | 2.7   | 2.7   | 2.1    | 0.3    | ---      | .6  | .13    |
| CO/hens bearing tumors                        | 2.1   | 2.8   | 2.4   | 2.1    | 0.3    | 0.5      | .4  | .27    |
| micrograms of non-hemoglobinous iron per gram |       |       |       |        |        |          |     |        |
| Normal Hens                                   | 2.1   | 3.6   | 10.5  | 5.2    | 0.9    | ---      | --- | .90    |
| Tumor-bearing Hens                            | ---   | ---   | 9.1   | 6.0    | 0.9    | 1.3      | --- | .50    |
| Copper / Iron Ratio                           |       |       |       |        |        |          |     |        |
| Normal Hens                                   | 1.1   | 0.9   | .34   | .40    | .33    | ---      | --- | .13    |
| Tumor-bearing Hens                            | ---   | ---   | .35   | .34    | .33    | .23      | --- | .50    |
| grams of tissue per Kilogram of chicken       |       |       |       |        |        |          |     |        |
| Normal Hens                                   | 2.3   | 3.1   | 22    | 7.1    | ---    | ---      | --- | ---    |
| Tumor-bearing Hens                            | 2.5   | 2.4   | 29    | 6.8    | ---    | ---      | --- | ---    |
| Carbon Monoxide Hens                          | 2.5   | 3.2   | 21    | 7.0    | ---    | ---      | --- | ---    |

\* Necrotic regions



carbon monoxide. The marked decreases observed in the heart and liver of the carbon monoxide chickens represent, therefore, an actual loss of copper from these organs, coincident with the changes in metabolism induced. The increased copper content of the plasma was offset by a diminished plasma volume, occasioned by a rise of 100 per cent in the erythrocyte content of the blood.

The decreased copper concentrations observed in the livers of the sarcomatous chickens were similarly compensated by an increased volume of liver tissue. The slightly decreased levels in the heart were accentuated by decreases in the volume of that organ. Similar effects have been observed in rabbits maintained upon a goitre-producing diet and in rabbits given the goitrogenic acetonitrile.<sup>27</sup> It cannot be too strongly emphasized that these changes in concentration and total content of catalytic copper and iron are the probable precursors of pathologic changes in organic structure.

The increased copper levels in the plasma of tumor-bearing chickens parallel the increases observed in the blood plasma of man in advanced carcinoma.<sup>25, 28</sup> The plasma and liver changes observed in the tumor-bearing chickens maintained in air containing carbon monoxide appear to represent a summation of the effects of tumor growth and of carbon monoxide.

The copper content of the tumor tissue itself varied from minimum, equivalent to that found in the surrounding muscle, to a maximum, observed in tumors containing more extensive regions of necrosis, of double the value found in muscle. Less copper has been observed in tumors in man than in the adjoining, non-malignant tissue.<sup>29</sup> It is highly probable that the amount of catalytically active copper present in growing, non-degenerating

Rous sarcoma tissue may be even less than the observed minimum for the total copper present.

Because of progressive changes in method and of the great experimental and analytical difficulties involved, fewer values were available for non-hemoglobinous iron than for copper. Those reported are representative but are to be regarded as indicative rather than final. The Rous sarcoma appeared to contain half as much again of non-hemoglobinous iron as the muscle in which it grew. The tumor contained much more total iron than muscle because of the presence of small areas of occluded material derived from blood. The tumor cells, and nuclei, have been reported, also, to contain an increased amount of undifferentiated iron.<sup>30</sup>

Additions of iron to cultures of Cl. sporogenes are followed by a marked stimulation of metabolism.<sup>24A</sup> The stimulation is not, of course, the result of any activation of molecular oxygen since none is available in an anaerobic culture. Additions of iron to aerobic cultures of yeast are followed by a decrease in oxygen uptake and a decreased capacity to reduce methylene blue,<sup>31</sup> -- evidences of a decreased capacity to utilize molecular oxygen as a source of energy. Additions of copper to cultures of properly prepared yeast are followed by heightened respiration and the practically complete cessation of anaerobic carbon dioxide production<sup>32</sup> -- evidences of an increased capacity to utilize molecular oxygen as a source of energy.

In passing from the brain and heart with their copper: iron ratio of 1:1 to the liver with its ratio of 1:3, one passes from tissues whose metabolic processes are concerned chiefly with the support of respiration to a tissue whose metabolic processes are used for purposes other than the exclusive support of



respiration. As one passes, further, to the Rous sarcoma with the ratio, less clearly defined because of analytical difficulties, of 1:4, one encounters a situation similar to that met in the iron-enriched yeast -- of decreased adaptation for the utilization of molecular oxygen as a source of energy. The tissue glycolyses even in the presence of air<sup>33</sup> and does not reduce methylene blue.

In normal tissue the iron levels are held down by a continuous burning-out of the iron catalysts by the respiratory process.<sup>25</sup> The impossibility of maintaining a normal iron level in cells where this regulating mechanism can no longer operate is apparent. The defect becomes self-perpetuated.

### III

#### The Comparative Copper and Iron Contents of the Tumor-Inducing and so-called Tumor-Inhibiting Fractions of Extracts of the Rous

##### Sarcoma

The extracts were prepared from tumor tissue as free as possible of necrotic material. The tissue was ground with special, acid-washed, metal-free sand and the finely divided mass triturated with Ringer's solution to a concentration of 10 per cent. The extracts could not be passed through a Berkefeld filter because of the (relatively) large amounts of metal which are dissolved from the filter and fittings during such filtration. They were rendered as nearly cell-free as possible by repeated centrifugation at 1700 r.p.m.

The clear supernatant from this centrifugation was carefully adjusted with metal-free 0.01 N  $H_2SO_4$  to pH 4.0-4.2,

bromo-cresol green being used as the indicator.\* The resulting precipitate was separated by centrifugation. Extracts of this precipitate produced tumors upon injection into chickens. Injections of the supernatant produced tumors only when the precipitate had been incompletely separated.

The concentrations of copper and iron in 10 per cent extracts of tumor tissue, in the still more dilute acidification supernatants, and in the purified tumor-inducing fractions were so low that accurate determinations with the methods available at that time were impossible. The few exploratory determinations made indicated that there can be little question but that the greater part of the copper content of the extracts remains with the tumor-inhibiting fraction upon acid fractionation, while iron accompanies the tumor-inducing fraction. It is planned to reopen the investigation of the metal content of the fractions within the near future. If the tumor-inducing principle does contain a disproportionate amount of active iron, it is easy to see how, after penetration into muscle tissue in an undenatured condition, it can accomplish a lowering of the copper:iron ratio and the induction of the more anaerobic metabolism which characterizes tumor tissue.

#### IV

#### The Effect of Respiratory Poisons on the Potency of Extracts of the Rous Sarcoma

Extracts of the Rous sarcoma rapidly lose their potency when kept in contact with air. Additions of cysteine retard this

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\*Robash, D. O., and Locke, A., Arch. Pathol. 11:835, 1931. The procedure was suggested by report of Murphy, J. B., Helmer, O. M., and Sturm, E., Science 68:18, 1928. The improved procedure of Sittenfeld, M. J., and Johnson, B. A., (Prac. Soc. Exper. Biol. & Med. 28:206, 1930) appeared after the work was already in progress.

deterioration provided the extracts are protected from further absorption of air by a covering of petrolatum.<sup>35</sup> Early in this investigation, we found that sodium cyanide, also, could be used as a preservative, an observation which had been duplicated, apparently, in the laboratory of Gye and Purdy.<sup>36</sup> Holmes and Pirie, later, found cyanide the better of the two preservatives for use under aerobic conditions.<sup>37</sup> Shibuya had shown, in 1929, that cultures of sarcoma cells, in vitro, could withstand concentrations of cyanide rapidly fatal to normal tissue.<sup>37A</sup>

Locke and Main differentiate the neurotoxins from the hemotoxins, the oxidases from the dehydrogenases, and the tryptic type proteases from the catheptic type on a basis of resistance to inactivation by cysteine and cyanide.<sup>24B</sup> The tumor-inducing agent of the Rous sarcoma is, under their classification, a diffusible respiratory substance, derived from the parent cell, carrying negatively charged, reducing iron and capable, on entrance into a living, growing cell "of orienting the synthesis of new respiratory substance in such a way that its own character is perpetuated rather than the character intrinsic to the cell to which it has gained access."

Glucolysis, definitely motivated by anionic, ferrous iron, is not inhibited by carbon monoxide.<sup>38</sup> It is inhibited by nitric oxide<sup>38</sup> and by flucrids.<sup>39</sup> We have found no retarding effect on the part of any of these substances on the tumor-inducing potency of Rous sarcoma extracts. Carbon monoxide, if anything, appeared to accelerate tumor growth (section V). Any inhibiting effect manifested by nitric oxide or fluoride may have been reversed by loss of inhibitor before entrance of the tumor-inducing agent into the tissues since the tissues could not be first saturated with the reagents tested as was possible with carbon monoxide.



No manifest inhibition of potency was observed in extracts containing antiseptic concentrations of phenol.

V

The Effect of Carbon Monoxide on the Resistance of Chickens  
to Inocula of Rous Sarcoma #1

Reports have appeared by Warburg, Wind and Negelein,<sup>40</sup> Campbell and Cramer,<sup>41</sup> and Sundstroem and Giragossintz<sup>42</sup> of a recession of tumor growth in rats kept in an atmosphere of low oxygen tension.

Table II summarizes the results of a series of comparisons of the growth of the Rous sarcoma in chickens kept in an atmosphere containing carbon monoxide and in chickens kept in an atmosphere free of carbon monoxide. Pure-bred Barred Rock pullets were used. Those in the first group were kept in a chamber of 300 liters capacity, supplied with approximately 500 liters per hour of air under positive pressure, containing 0.05 to .07% of carbon monoxide prepared according to the directions of Nicloux.<sup>43</sup> The chickens used as controls were kept in a chamber constructed and ventilated exactly like the gas chamber. Both the air and gas chambers were made as comfortable as possible by the presence of absorbents for moisture and carbon dioxide and by large glass windows. The chickens placed in the gas chambers were gradually acclimated to the final concentration of carbon monoxide maintained. Frequent determinations of the carbon monoxide content of the chambers, and of the blood of the chickens kept there, were made by the method of Sayers and Yant.<sup>44</sup>

The chickens maintained in carbon monoxide had from 80 to 100% more hemoglobin in their circulating blood than the controls. Parallel increases in the amount of hemoglobin deposited in the tissues were also apparent in the deeper pigmentation of

TABLE II

THE INEFFECTIVENESS OF CARBON MONOXIDE IN INCREASING THE  
RESISTANCE OF THE PECTORAL MUSCLE OF HENS TO INVASION  
BY THE TUMOR-INDUCING AGENT OF ROUS SARCOMA #1

| Weight of<br>hens<br>injected,<br>gms. | Days kept<br>in CO<br>chambers<br>before<br>injection | Lot number<br>of tumor<br>extract<br>injected | Size of<br>tumor<br>produced | Days survival<br>after the<br>injection |
|----------------------------------------|-------------------------------------------------------|-----------------------------------------------|------------------------------|-----------------------------------------|
|----------------------------------------|-------------------------------------------------------|-----------------------------------------------|------------------------------|-----------------------------------------|

Chickens kept in air containing .04-.07 % of carbon monoxide

|     |     |     |       |      |
|-----|-----|-----|-------|------|
| 1.2 | 3-4 | 1   | large | 17   |
|     |     | 1A* | small |      |
| 1.2 | 3-4 | 2   | large | 29   |
| .5  | 8   | 3   | large | 20** |
|     |     | 3A* | large |      |
| .5  | 8   | 4   | large | 20** |
|     |     | 4A* | large |      |
| 1.2 | 32  | 5*  | large | 19   |
| .7  | 25  | 7*  | large | 15   |
| .7  | 25  | 8*  | large | 14   |

Control Chickens that were kept in carbon monoxide-free air

|     |  |     |       |    |
|-----|--|-----|-------|----|
| 1.2 |  | 1   | large | 39 |
|     |  | 1A* | none  |    |
| 1.2 |  | 2   | large | 39 |
| 1.2 |  | 5*  | large | 26 |
| 1.2 |  | 5*  | large | 28 |
| .7  |  | 7*  | large | 21 |
| .7  |  | 8*  | large | 15 |

\*Presumably cell-free, passed through a Berkefeld  
filter N before injection.

\*\*Killed.

the surface musculature. The increases were compensatory in nature, tending to balance the diversion, by the carbon monoxide absorbed, of some 40 to 50% of the normally oxygen-carrying pigment. In spite of these compensating actions, the oxygen tension available to the tissues of the carbon monoxide chickens was, necessarily,<sup>45</sup> lower than that available to the tissues of the controls.

The tumors of the carbon monoxide chickens grew fully as large and possibly more rapidly than those of the controls. The chickens kept in the carbon monoxide chamber died from one to two weeks sooner than the controls, possibly because of an aggravation of their condition by the carbon monoxide itself.

The discrepancy between these results and those reported by the investigators named in the opening paragraph has been corroborated in the paper of J. A. Campbell published after the experimental work of our own investigation had been completed. Campbell found no effect of carbon monoxide on the growth of the Rous sarcoma in chickens but observed a marked retardation by carbon monoxide on the growth of a transplantable carcinoma of the mouse.<sup>46</sup>

Carbon monoxide is without effect on glucolysis<sup>38</sup> and proteolysis.<sup>27</sup> It does not inhibit the respiration of certain of the cytochrome-free aerobic molds; it does inhibit the respiration of molds budded from the same initial strain but grown in such a way as to contain cytochrome.<sup>21</sup> The Rous sarcoma contains no cytochrome.<sup>47</sup> Carcinomas transplantable in rats and mice do, although not to the extent found in normal tissue.<sup>47</sup> The contradictory findings on the effects of carbon monoxide on tumor growth in the chicken and the rat probably find, in these facts, their explanation.



VI

The Antitrypsin content of the Serum and Tissues of Normal  
and Tumor-Bearing Chickens

Fresh, unheated blood serum inhibits the proteolytic action of trypsin to an extent determined by the proportionate amounts of serum and trypsin interacting. This property, whether it derives from the presence in the serum of a specific antitryptic principle or not, is of definite interest because of the characteristic changes which are observed in disease. The trypsin-inhibiting potency of the serum has been found by Locke and Main to parallel, roughly and with exceptions, the copper levels observed.<sup>48</sup> Marked increases are found in cancer.

Table III compares the antitryptic activity of blood serum from normal chickens with that from chickens bearing well-developed growths of the Rous Sarcoma #1. The titrations were made according to the unpublished directions of Locke and Main which are as follows:

"Exactly 0.2 cc. of the serum sample is diluted to 1.6 cc. with a phosphate buffer of pH 8.0. Measurements of 0.48, .32, .24, .16, and 0 cc. of this dilution are made into a series of marked 5x5/8" test tubes. A similarly prepared 1:8 dilution of beef serum which has been heated, slowly, to 85°C. and subsequently cooled before use is then added to the tubes in amounts sufficient to give a total volume of 2.4 cc. The contents of the tubes are mixed and decanted as completely and uniformly as possible into correspondingly marked 6x3/4" test tubes containing, each, 0.1 cc. of a 1% suspension, in pH 8.0 buffer, of Pfanstiehl trypsin. After careful mixing, the contents are covered with a thin layer of toluene; the tubes are immersed in water adjusted to 38°C. and held at that temperature for 16 hours.

"At the end of this incubation period, a 2.0 cc. sample is withdrawn from each tube and mixed with an equal volume of 20% trichloroacetic acid. The resulting precipitate is removed by filtration and the N content of the clear filtrate determined by the method of Folin and Denis.

"For the calculation of the per cent inhibition of tryptic activity, the observed N contents are corrected by the deduction of a blank value characterizing the non-

TABLE III

COMPARATIVE TITRATIONS OF THE ANTITRYPTIC ACTIVITY OF THE BLOOD  
SERUM OF NORMAL AND TUMOR-BEARING CHICKENS

| Volume of<br>antitryptic<br>serum<br>present,<br>cc. of<br>1 : 8<br>dilution | mg. of non-precip-<br>itable N per cc.<br>of incubated mix-<br>ture.* Serum from: |                              | Indicated inhibition<br>of tryptic activity |                  |             |                  |
|------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------|---------------------------------------------|------------------|-------------|------------------|
|                                                                              | normal<br>chickens                                                                | sarcom-<br>atous<br>chickens | in mg. NPN                                  |                  | in per cent |                  |
|                                                                              |                                                                                   |                              | normal                                      | sarcom-<br>atous | normal      | sarcom-<br>atous |
| 0.48                                                                         | 0.35                                                                              | (0.15)                       | 0.8                                         | (1.0)            | 70          | (90)             |
| .32                                                                          | .55                                                                               | .25                          | .6                                          | .9               | 50          | 80               |
| .24                                                                          | .80                                                                               | .35                          | .35                                         | .8               | 30          | 70               |
| .16                                                                          | (.95)                                                                             | .55                          | (.2)                                        | .6               | (20)        | 50               |
| 0                                                                            | 1.15                                                                              | 1.15                         | ----                                        | ----             | ----        | ----             |

\*  
After deduction of a blank of 0.11 mg.

precipitable N content of the mixtures before incubation. The corrected values are subtracted from the corrected value for the control tubes (containing 0 cc. of unheated serum dilution) and the differences recorded as the extent of inhibition in terms of mg. of N. The per cents of inhibition are the quotients of the latter values by the corrected N of the control tube filtrates.

"The unit of antitryptic activity is reported as the volume of serum which inhibits the activity of 1 mg. of Pfanstiehl trypsin by 50%."

Blood serum from normal chickens appears to contain from 25 to 30 units of antitryptic activity per cc., as compared with the titer of approximately 40 found in man. Serum from chickens with well-developed tumors appears to contain from 50 to 60 units per cc., an increase comparable to that observed in man in advanced carcinoma.

Table IV compares the antitryptic activity of a series of Ringer's solution extracts of sand-ground tissues from normal and tumor-bearing chickens. Extracts made from the liver, brain, spleen, and muscle were found to contain little or no antitryptic activity. Similarly prepared extracts of the kidneys contained an average of 3 units per gram of tissue in the normal chicken, and an average of 5 units per gram in the tumor-bearing chicken. Extracts of the non-necrotic portions of the tumors contained from 5 to 6 units per gram; extracts of the necrotic portions, from 6 to 7 units per gram.

The trypsin-inhibiting activity of extracts of the Rous sarcoma, while small in comparison with that observed in the blood serum, is, nevertheless, significant because of the fact that extracts of the tissue in which the tumor grows are devoid of such activity. The presence of slightly increased concentrations of antitrypsin in the necrotic portions of tumors, the marked increases in the blood, and the parallel increases in the kidneys in sarcoma-carrying chickens would appear to suggest that



TABLE IV

COMPARISONS OF THE ANTITRYPTIC ACTIVITY OF EXTRACTS OF THE  
TISSUES OF NORMAL AND TUMOR-BEARING CHICKENS

| Tissue<br>Extracted*                | mg. of non-precip-<br>itable N per cc.<br>of incubated mix-<br>ture** |                              | Indicated inhibition<br>of tryptic activity |                  |             |                  |
|-------------------------------------|-----------------------------------------------------------------------|------------------------------|---------------------------------------------|------------------|-------------|------------------|
|                                     |                                                                       |                              | in mg. NPN                                  |                  | in per cent |                  |
|                                     | normal<br>chickens                                                    | sarcom-<br>atous<br>chickens | normal                                      | sarcom-<br>atous | normal      | sarcom-<br>atous |
| Liver,<br>heated:                   | 0.66                                                                  | 0.76                         |                                             |                  |             |                  |
| unheated:                           | .66                                                                   | .76                          | ∅                                           | ∅                | ∅           | ∅                |
| Spleen,<br>heated:                  | .73                                                                   | .55                          |                                             |                  |             |                  |
| unheated:                           | .68                                                                   | .45                          | 0.05                                        | 0.10             | 7           | 18               |
| Brain,<br>heated:                   | .68                                                                   | .64                          |                                             |                  |             |                  |
| unheated:                           | .70                                                                   | .60                          | ∅                                           | .04              | ∅           | 7                |
| Muscle,<br>heated:                  | .60                                                                   | .63                          |                                             |                  |             |                  |
| unheated:                           | .56                                                                   | .60                          | .04                                         | .03              | 7           | 5                |
| Kidney,<br>heated:                  | .63                                                                   | .64                          |                                             |                  |             |                  |
| unheated:                           | .48                                                                   | .30                          | .15                                         | .34              | 25          | 50               |
| Tumor, non-<br>necrotic,<br>heated: |                                                                       | .54                          |                                             |                  |             |                  |
| unheated:                           |                                                                       | .22                          |                                             | .32              |             | 60               |
| Tumor,<br>necrotic,<br>heated:      |                                                                       | .46                          |                                             |                  |             |                  |
| unheated:                           |                                                                       | .14                          |                                             | .32              |             | 70               |

\*After correction for blank values.

\*\*Equivalent amounts were compared, incubated with, as  
nearly as possible, equivalent amounts of substrate.

the substance may be a product of cellular disintegration, in process of continuous, slow elimination. Increases in the minute and almost inestimable content of antitrypsin in the urine have been reported in diseases characterized by an increased antitryptic titer in the blood serum.<sup>49</sup>

Table V is an outline of the method used for the concentration of the antitrypsin present in extracts of the Rous sarcoma. Adjustment of the pH of the extracts to 4.0-4.4 was followed by the separation of a protein-like precipitate carrying the tumor-inducing principle. The precipitate carried with it little or no antitrypsin. The latter principle remained in the supernatant from which it was separated by saturation with sodium chloride. Extraction of the salt precipitate with pH 8 phosphate buffer, followed by the addition of two volumes of 95% alcohol and the removal of the resulting precipitate, accomplished an almost complete separation from protein without diminution of antitryptic activity. The final preparations were slightly viscous, with a light yellow color. They contained no hematin.

Substances soluble in aqueous alcohol and capable of inactivating trypsin have been isolated from trypsin digests of gelatin and casein by Northrup.<sup>50</sup> They may be produced by the tissues, in conditions analogous to cachexia, and, perhaps, normally to a less degree, as the result of tryptic self-digestion, or they may be produced as the result of the macrophagic disintegration of injured cells.

## VII

### The Absorption Spectrum of a Green Pigment Present in Some of the Tumor-Bearing Chickens

A green pigment is often found in chickens bearing the Rous sarcoma when the growth of the tumor is extensive. The

TABLE V

THE CONCENTRATION OF THE ANTITRYPTIC ACTIVITY OF EXTRACTS OF THE  
ROUS SARCOMA

| 100 cc. of average extract                                                                             |                                                                                                                                                                                                  |
|--------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| mg. N present, 39; units antitrypsin, 57; units antitrypsin per<br>mg N, 1.46. Adjusted to pH 4.0-4.4. |                                                                                                                                                                                                  |
| Precipitate,<br>(tumor-inducing fraction)<br>Units antitrypsin present,<br>Ø                           | Supernatant,<br>(so-called tumor-inhibiting fraction)<br>mg.N present, 13; units antitrypsin,<br>54; units antitrypsin per mg.N, 4.2.<br>Saturated with NaCl and centrifugated<br>after 2 hours. |
| Supernatant,<br>Units antitrypsin present,<br>Ø                                                        | Precipitate,<br>Shaken with pH 8 phosphate buffer for<br>20 min. at room temperature.                                                                                                            |
| Residue,<br>rejected                                                                                   | Extract,<br>mg.N present, 2.6; units antitrypsin,<br>41; units antitrypsin per mg. N, 16.<br>Diluted with 2 volumes of absolute<br>alcohol.                                                      |
| Precipitate,<br>rejected                                                                               | Supernatant,<br>mg.N present, 1.05; units antitrypsin,<br>33; units antitrypsin per mg. N, 31.                                                                                                   |



pigment is usually localized in the interstices between the skin and muscles; often the tissues themselves are colored a pale green. Since the present investigation centered around the respiratory catalysts of the tissues and since the color of the pigment suggested the possible presence of a porphyrin moiety, an attempt was made toward its identification.

No earlier references were found to the occurrence of this pigment in tumor-bearing chickens. The appearance of a green pigment in chlorosis and occasionally in carcinoma in man has been attributed to the formation of a sulfhemoglobin by the action on hemoglobin of the hydrogen sulfide liberated by autolyzing tissue. Warburg and Negelein have reported the formation of a green hemin through the action of a reducing agent on red hemin in the presence of oxygen. The absorption spectrum of the green hemin consisted of three bands, the largest between 645-680  $\mu\mu$  and the smaller and less intense bands in the lower portion of the visible spectrum.<sup>52</sup> Very recently, Lemberg and Barcroft have reported the isolation of a green pigment from the placentas of dogs. This pigment was similar to biliverdin both in its absorption spectrum and in its reactions with the Gmelin reagents. Solutions of the pigment in methyl alcohol gave a single absorption band occurring at 640-680  $\mu\mu$  with a maximum at approximately 650  $\mu\mu$ .<sup>52</sup>

Pyridine is known to be a good solvent for the porphyrins, and was, therefore, used for the extraction of the green pigment from chicken tissue. The resulting extracts, when centrifugated, gave a supernatant brilliantly clear and bluish-green in color. The absorption spectrum in the visible range showed the presence of a single band at 645-680 with a maximal absorption occurring at 360. The compound was evidently not sulfhemoglobin, and bears

more than a casual resemblance to the "green hemin" of Warburg and Negelein and the placental pigment of Lemberg and Barcroft. While Lemberg and Barcroft were unable to demonstrate the presence of a pigment-producing extracellular enzyme, we have found that tissues do form the green pigment *in vitro*, providing a suitable medium is used.

When tissues are placed in pyridine for the extraction of the green pigment, the red color of any adhering blood soon becomes, also, dark and green. Immersion of several pieces of sarcoma tissue in pyridine containing rabbit hemoglobin resulted in a gradual darkening of the color of the solution and the appearance of a green pigment in the solution. This change occurred both at room temperature and at 37.5° C. Muscle tissue behaves similarly but the rate of formation of the green pigment was slower and the amount produced less. Liver, kidney, and spleen, also, were able to convert hemoglobin into the green pigment. These tissues will not form the pigment in ether or alcohol nor is a solution of hemoglobin alone in pyridine converted into the pigment in the presence of air and sunlight.

The formation of the pigment in the tissues may be explained by the presence of a compound capable of stabilizing the iron of hemoglobin in the ferrous condition while the porphyrin molecule is being oxidized by the oxidases of dying cells. Pyridine has been found to stabilize iron in the ferrous condition *in vitro*.<sup>53</sup>

## VIII

### Summary

A relation has been established between the concentration and proportion of copper and non-hemoglobinous iron in the tissue of normal and tumor-bearing chickens and the intensity and

balance of the metabolic processes supported. The highest concentrations of copper and non-hemoglobinous iron observed in the tissues examined were in the liver; the lowest in the inactive breast muscles. The highest copper/iron ratios were observed in the brain and heart, tissues whose metabolism is devoted more exclusively to the support of respiration than is the case in the liver, kidney, and muscle, tissues characterized by lower ratios. The lowest ratio observed was in the tumor material itself, where glucolysis persists even in the presence of air and methylene blue is not reduced.

Exploratory fractionations of potent extracts of the Rous sarcoma have indicated the possibility that copper may accompany the tumor-inhibiting fraction, while iron may accompany the tumor-inducing fraction. It is suggested that a normal muscle cell into which an iron-carrying, tumor-inducing principle had diffused would have an altered copper/iron ratio, characteristic of tumor tissue rather than of normal muscle tissue.

The potency of tumor-inducing extracts is not impaired following contact with the respiratory poisons: cysteine, sodium cyanide, carbon monoxide, nitric oxide, sodium fluoride, or phenol. Tumor extracts, injected into chickens kept in air containing carbon monoxide for a period sufficiently long to produce changes in the hemoglobin content of the blood and in the pigmentation of the musculature, produce tumors fully as large and, perhaps, more rapidly growing than when injected into chickens kept in air free of carbon monoxide.

The trypsin-inhibiting capacity of the blood serum of chickens carrying well-developed tumors is double that of normal chicken serum. The order of antitryptic activity observed in extracts of the tissues of normal and tumor-bearing chickens was

as follows: necrotic regions of tumor tissue > non-necrotic regions > kidneys of tumor-bearing chickens > kidneys of normal chickens. Little or no activity was observed in extracts of liver, brain, spleen, and muscle. A method for the concentration of the anti-tryptic principle is described and suggestions made as to its nature.

A green pigment was observed in many of the tumor-bearing chickens, analogous in its absorption spectrum to the "green hemin" of Warburg and Negelein and to the placental pigment of Lemberg and Barcroft. It is not sulphemoglobin as has been commonly believed.

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